ORIGINAL ARTICLE

Superoxide production by peripheral polymorphonuclear leukocytes in patients with COPD

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Abstract Polymorphonuclear leukocytes (PMNs) have been implicated in the pathogenesis of COPD, partly because of the release of oxidants, like superoxide anion (SA). The goal of this study was to measure the spontaneous and stimulated release of SA by peripheral PMN in stable COPD compared with healthy controls. Seventeen patients with stable moderate COPD and 17 healthy age-matched controls were included. SA release from peripheral PMN was measured spectrophotometrically as the superoxide dismutase (SOD) inhibitable reduction of cytochrome c. PMNs were stimulated with phorbol myristate acetate (PMA, 1 and 10 ng/ml), diesel exhaust particles (DEPs), carbon black (CB) and ultrafine CB (ufCB, 125, 250 and 500 μg/ml). The spontaneous SA release (PMA-0) between patients and control subjects was not significantly different. After stimulation with PMA, SA release increased in both patients and controls. The SA release did not increase after stimulation with DEP and CB in patients nor in controls. There was only an increase after stimulation with ufCB in the patient group. The increased SA release in COPD patients after stimulation with ufCB may suggest that PMN of COPD patients are more prone to stimulation and that the smaller particle size of ufCB might be a crucial factor.

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Keywords COPD; polymorphonuclear leukocyte; PMN; neutrophil; superoxide anion; particulate air pollution.

INTRODUCTION

Polymorphonuclear leukocytes (PMNs) have been implicated in the pathogenesis of COPD. They are involved in the inflammatory process, reflected by an increased number of neutrophils in sputum, bronchoalveolar lavage fluid (BAL fluid) and bronchial biopsies in smokers and COPD patients (1–5). In addition, a large pool of PMNs is present within the microvessels of the lung (6).

Following a stimulus (e.g. infection, injury, inhalation of smoke and particulate matter), PMNs migrate from the peripheral blood into the extravascular space. Some migrate to the lungs and through the endothelium into the airway lumen, where they perform their 'task' of phagocytosis and destruction of an intruding organism or particle (7,8). Bosken *et al*. showed that the number of submucosal PMNs is related to the amount smoked (4). A possible mechanism is that alveolar macrophages of smokers release a chemotactic factor for neutrophils (5).

Eventually, the accumulation and activation of PMN may result in an adverse effect of destruction of parenchymal tissue (i.e. development of emphysema), both through proteolysis by the elastase released from the lysosomal granules and the release of reactive oxygen-derived species (ROS), like superoxide anion (SA) and hydrogen peroxide (H_2O_2). *In vivo* evidence for an increased oxidative burden has been provided by Dekhuijzen *et al.* who showed that patients with stable COPD had higher concentrations of H_2O_2 in exhaled breath

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condensate than healthy control subjects (9). The production of H_2O_2 was further increased during exacerbations.

The increased oxidative burden is not only determined by an increase in the number of inflammatory cells like PMN, but also by an increased production of oxidants per cell (10). In healthy smokers, PMN showed increased production of oxidants (11). In COPD patients, Teramoto *et al*. showed that spontaneous production of oxidants (SA) by PMN was similar to normal subjects. In contrast, Noguera *et al*. showed an increased production of oxidants by PMN in COPD patients compared with smokers with normal lung function (12,13). Two studies showed that the production of oxidants by PMN was increased after stimulation with phorbol myristate acetate (PMA) in COPD patients compared with healthy smokers (12,13). The production was also higher in smokers compared with non-smokers (11).

Elevated concentrations of respirable particulate [i.e. fine particles with a diameter less than 10 μm in diameter (PM_{10})] have been implicated in adverse health effects, including COPD (14,15). Respirable particulates contain a variety of toxic substances on their surface and arise amongst others from motor vehicle exhausts, especially those emitted from diesel engines (16). These diesel exhaust particles (DEPs) contain both dust (elemental carbon), traces of heavy metals and organic components, which both enhance production of ROS by alveolar macrophages *in vitro* (17,18). In animal studies, intratracheal instillation of DEP, carbon black (CB) and ultrafine particulate (ufCB), caused an acute inflammatory response with an influx of PMN in alveolar air spaces and interstitium (17–19). Respirable particulate can also be used to stimulate PMN directly.

The effects of stimulation with respirable particulate on SA generation by PMN of COPD patients *in vitro*, are not known. Therefore, the aim of the present study was to compare the release of SA by peripheral PMN, both spontaneously and after stimulation with PMA, DEP, CB and ufCB between stable COPD patients and healthy controls.

METHODS

Subjects

Seventeen patients diagnosed with (stable) COPD according to the criteria of the ERS (20) participated in this study. Inclusion criteria were: age <75 years, current or ex-smoker, forced expiratory volume in 1 s (FEV_1) between <70 % of predicted or FEV_1/FVC <60 % of predicted and reversibility less than 15% of predicted after inhalation of 400 μg salbutamol or 80 μg ipratropium bromide. Further, 17 healthy age-matched control subjects were recruited. Exclusion criteria for both healthy controls and patients were a history of asthma, allergy, atopic constitution, known α_1-proteinase inhibitor deficiency or other pulmonary diseases, an exacerbation or cold within the previous 8 weeks and use of supplemental dietary anti-oxidants, vitamins or N-acetylcystein. Both control subjects and patients were considered to be ex-smokers if they had discontinued smoking for at least 1 year prior to the study. This study was approved by the local Ethical Committee and informed consent was obtained from all subjects.

Sample collection and preparation

From each participant, 30 ml of venous lithium-heparinized blood was collected at 16.00 h. Samples were kept overnight at room temperature in the dark and prepared according to Schalkwijk *et al.* (21) at the next day at 08.00 h. After centrifugation, plasma was removed and the buffy coat diluted 1:1 with physiological saline. Subsequently, 8 ml of the plasma expander Plasmasteril was added for sedimentation of the red cells.

The dextran plasma layer, containing plasma, lymphocytes and monocytes was collected and 7 ml of Ficoll–Hipaque solution (Pharmacia Biotech) with a density of 1.070 g/ml was gently brought under the supernatant. After centrifugation at room temperature during 20 min at 2000 rpm, the plasma, lymphocytes, monocytes and Ficoll–Hipaque were removed. The remaining PMNs (density 1.080 g/ml) and erythrocytes (density 1.085 g/ml) in the cell pellet were suspended in 0.5 ml Hanks' balanced salt solution (HBSS), and 13 ml of aqua pure was added to lysate the erythrocytes. After centrifugation at 4°C during 5 min at 1000 rpm, the cell pellet was washed with HBSS and finally resuspended in 2.5 ml HBSS + 0.1% glucose.

The total and differential cell counts were performed with a standard hemocytometer. Viability was assessed with the trypane blue exclusion test. The yield of PMN was 30–40 % of the first sample with a high percentage of neutrophils [mean (SEM) 92.4 % (0.37)] and a viability of 98% or more.

Superoxide assay

SA release was measured spectrophotometrically as the superoxide dismutase (SOD) inhibitable reduction of cytochrome *c*, adapted for 96-well assay. Briefly, 25 μl of HBSS was added to 50 μl of cytochrome *c* (640 μM, Sigma Chemicals), 25 μl of SOD (2400 U/ml, Sigma Chemicals), 25 μl of PMN suspension (4×10^6/ml) and 50 μl of stimulus. For measurement of spontaneous release no stimulus was added (PMA-0). For measurement of release, with (25 μl) and without SOD and with (50 μl) and without cells, respectively, the final volume of 200 μl was adjusted by adding HBSS.

Stimuli

PMA (Phorbol 12-Myristate 13-Acetate, Sigma Chemicals) was used as a stimulus in a concentration of 1 and 10 ng/ml. Samples were incubated during 30 min at 37°C. The extinction was measured every 2 min at 540 and 550 nm using a Softmax 96-wells microplate reader. Since there was a linear increase up to 30 min for both patients and healthy control subjects, the extinction after 30 min was used to calculate the concentration of reduced cytochrome with a extinction coefficient of 14 964 L/mol cm.

DEP were collected from a 4 kW diesel engine (Yanmar, L90E, single cylinder). DEP, CB (acetylene carbon black, Strem, Newsburyport, MA, U.S.A.) and ufCB (Printex-90, diameter 20 nm, surface area 33.7 m^2/g) were suspended in a final concentration of 2 mg/ml phosphate-buffered saline with 0.05% Tween-80 after sonification (MSE 100 Watt, Ultrasonic Disintegrator 7100). Concentrations of 125, 250 and 500 μg/ml were prepared. After stimulation, reaction mixtures were incubated at 37°C during 30 min in a 24-well plate.

Since addition of these stimuli caused a black mixture, measurement of extinction in time was not possible. Therefore, samples were filtered after 30 min and extinction of the supernatant was measured in the 96-well microplate reader. The addition of 500 μg/ml ufCB to PMN probably caused a toxic reaction, since cells coagulated and SA release was diminished. Therefore, the results of stimulation with 500 μg/ml ufCB were disregarded.

All samples were prepared in duplicate and the average value of the extinction was used for calculation. Finally, the SOD inhibitable reduction of cytochrome *c* was calculated as the differences in extinction between the wells with and without SOD.

The results were expressed as the concentration of reduced cytochrome *c* (nmol/ml) per 1×10^6 PMN per 30 min.

Statistical analysis

Data are expressed as mean and standard error of the mean (SEM). Differences between patients with COPD and healthy controls were analysed with the Student's *t*-test for independent samples. Influence of smoking and use of medication was analysed by analysis of covariance. The slope of SA release with increasing concentration of the stimuli was calculated with the linear regression equation. Differences in the slope between groups were tested with the Mann-Whitney *U*-test using the individual slopes. Differences in the slopes within groups between similar stimuli were tested with the Friedman's analysis of variance for related samples. For all analyses, a *P*-value less than 0.05 was considered significant.

RESULTS

Subjects

Seventeen COPD patients [mean FEV_1 56.6 (4.9)% of predicted] and 17 healthy controls were included (Table 1).

Spontaneous SA release

The spontaneous SA release (PMA-0) between patients and control subjects was not significantly different [mean (SEM) 10.4 (1.4) *vs.* 10.3 (0.9) nmol/ml per 10^6 cells per 30 min, $P = 0.9$] (Fig. 1). Both smoking history and use of medication did not influence this result (data not shown). The concentrations of DEP-0, CB-0 and ufCB-0 in the patient group were 5.5 (0.9), 4.7 (0.8), and 4.2 (0.9) nmol/

Table 1. Characteristics study population

	Healthy controls (N = 17)	Patients (N = 17)
Age (years)	58 (2)	61 (2)
FEV_1 (% of predicted value)	na	56.6 (4.9)
Reversiblity (%)	na	6.8 (1.1)
Smoking history		
Never smokers	8	na
Ex-smokers	6	14
Current smokers	3	3
Medication		
ICS	na	11

Mean (SEM), FEV_1 = forced expiratory volume in 1 second; reversibility = change in FEV_1 after 400 μg salbutamol; na = not applicable; ICS = inhaled corticosteroids.

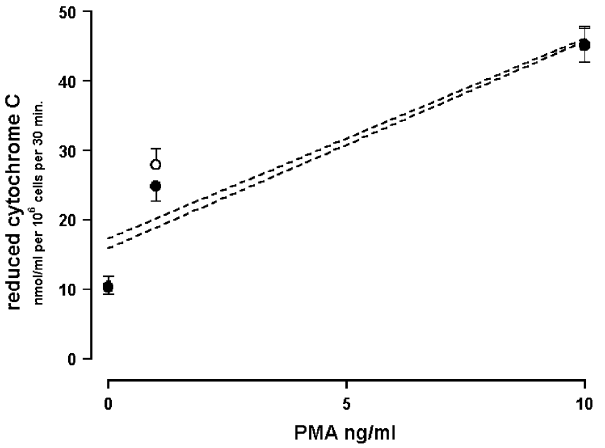

Fig. 1. Regression analysis of the concentration of PMA and the concentration of reduced cytochrome *c* (nmol/ml) per 10^6 cells per 30 min. Open circles (○) and error bars represent mean (SEM) values of healthy controls, solid circles (●) and error bars represent mean (SEM) values of patients.

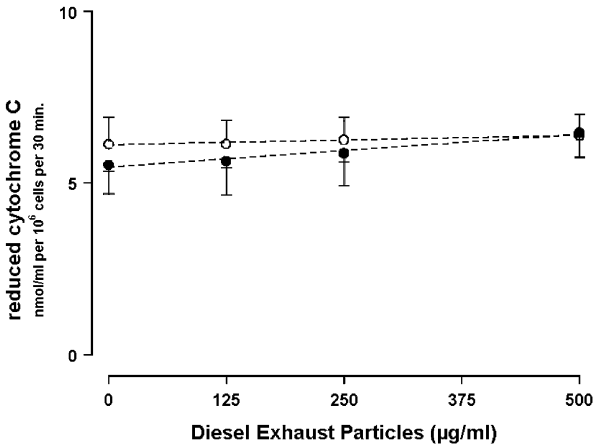

Fig. 2. Regression analysis of the concentration of DEP and the concentration of reduced cytochrome *c* (nmol/ml) per 10^6 cells per 30 min. Open circles (○) and error bars represent mean (SEM) values of healthy controls, solid circles (●) and error bars represent mean (SEM) values of patients.

ml 10^6 cells per 30 min, respectively. In the healthy control group, the concentrations were 6.1 (0.8), 6.3 (0.7), and 5.1 (0.7) nmol/ml 10^6 cells per 30 min, respectively. There were no significant differences between patients and healthy controls.

SA release after stimulation with PMA

The concentration of reduced cytochrome *c* per 10^6 cells per 30 min after stimulation with 1 ng/ml PMA (PMA-1) and 10 ng/ml PMA (PMA-10), increased significantly in both patients and control subjects ($P < 0.01$, $P < 0.01$, respectively) (Fig. 1). The mean concentration for PMA-1 was 24.84 (2.14) nmol/ml in patients and 27.9 (2.4) nmol/ml in healthy controls ($P = 0.34$). For PMA-10, the mean concentration was 45.1 (10.7) nmol/ml in patients and 45.3 (10.5) nmol/ml in healthy controls ($P = 0.95$).

No significant differences in SA release nor in the slope of increase in SA release were observed between patients and control subjects. Smoking and the use of ICS did not influence the results (data not shown).

SA release after stimulation with DEP, CB and ufCB

The mean concentrations of reduced cytochrome *c* per 10^6 cells per 30 min were similar in patients and healthy controls, and did not increase after stimulation with DEP (Fig. 2). After stimulation with CB a small increase in SA release was observed in patients (Fig. 3). Yet, the slope of increase in patients compared to controls was not significantly different. In contrast, after stimulation with ufCB, the slopes of increases were significantly different ($P = 0.02$) (Fig. 4), i.e. an increased SA release in patients

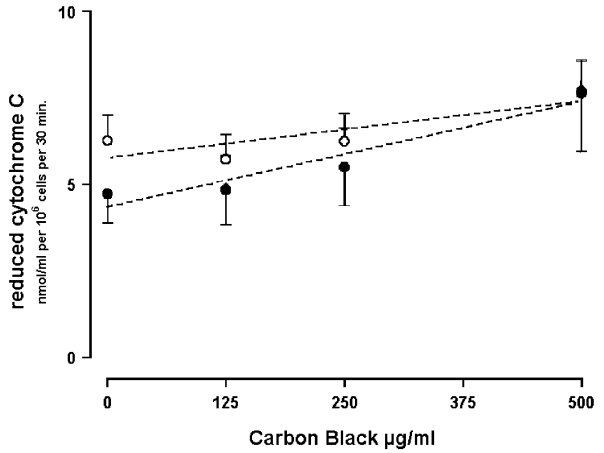

Fig. 3. Regression analysis of the concentration of CB and the concentration of reduced cytochrome *c* (nmol/ml) per 10^6 cells per 30 min. Open circles (○) and error bars represent mean (SEM) values of healthy controls, solid circles (●) and error bars represent mean (SEM) values of patients.

compared with healthy controls. Again, smoking history and the use of medication did not influence the results with respect to DEP, CB and ufCB (data not shown).

DISCUSSION

The present study shows that the spontaneous release of SA by peripheral PMN in COPD is not increased, compared with healthy control subjects. After stimulation with PMA, SA release was equally increased in both COPD patients and healthy controls. After stimulation with DEP and CB, the SA release was not increased in patients nor in healthy controls. Only after stimulation

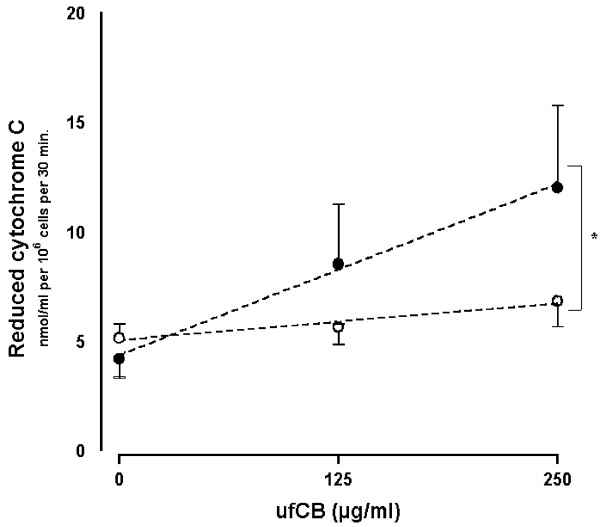

Fig. 4. Regression analysis of the concentration of ufCB and the concentration of reduced cytochrome *c* (nmol/ml) per 10^6 cells per 30 min. Open circles (○) and error bars represent mean (SEM) values of healthy controls, solid circles (●) and error bars represent mean (SEM) values of patients. *$P < 0.05$

with ufCB the SA release was significantly higher in COPD patients.

The results of the present study are in accordance with the study of Teramoto *et al.*, who also found no difference in spontaneous SA release between COPD patients and healthy controls (12). In contrast with Ludwig *et al.*, we observed no differences between smoking and non-smoking individuals (11). Postma *et al.* did not find a difference between smokers and ex-smokers with chronic airflow obstruction either (22).

In contrast to the present study, Noguera *et al.* found that the production of ROS by PMN was increased in patients with COPD compared with healthy smokers and non-smokers (13). However, they measured the ROS production by the NADPH oxidase method and not by SA release, which makes it difficult to compare results.

The use of ICS did not influence the results in the present study. Verhoeven *et al.* also found no effect of inhaled fluticasone propionate on superoxide production by pulmonary cells retrieved with BAL in smoking COPD patients (23).

The failure to demonstrate differences in spontaneous SA release may suggest that the rest status of PMN in stable COPD patients is not different from that in controls. However, SA is only one of the oxidants that is produced by PMN, they also produce H_2O_2 and hydroxyl radicals.

On the other hand, since formation of SA occurs prior to other members of ROS, differences in production of other oxidants by PMN at rest between COPD patients and healthy controls is very unlikely.

The concentrations of DEP-0, CB-0 and ufCB-0 were lower than the PMA-0 concentration. Since addition of these stimuli caused a black mixture, measurement of extinction in time was not possible. Therefore, samples were analysed as described in the method section, which may explain the difference in baseline value.

The data of the present study show that the release of SA by PMN after stimulation with PMA is equally increased in COPD patients and healthy controls. This is in contrast with the data presented by several other investigators (12,13,22).

As mentioned above it is difficult to compare our results with the results of Noguera *et al.* because of the difference in technique (13). The difference in result between our study and the study by Postma *et al.* could be due to the time of reaction (22). They measured the SA release after 15 min of reaction while in the present study the SA release was measured after 30 min. Indeed, the SA release was higher in our study using the same PMA concentration. It is possible that the difference in SA release between PMN of COPD patients and healthy controls present at 15 min disappears after a longer reaction time. Teramoto *et al*. measured the SA release in whole blood by chemiluminescence, since in their opinion this is more likely to reflect the *in vivo* situation (12). Unfortunately, comparison with the current data is difficult, primarily due to the different methodologies applied.

After stimulation with particles present in environmental air (DEP, CB and ufCB), SA release increased only after ufCB stimulation in COPD patients.

To our knowledge, the effect of stimulation with respirable particles on PMN of COPD patients has not been studied. It seems that exposure to DEP leads to accumulation of PMN, possibly through chemotactic agents, but not to increased production of oxidants by PMN (17,19). Only ufCB had an effect on the production of oxidants by PMN in COPD patients in this study. This effect may be explained by the smaller particle size of ufCB. Several studies have shown that ufCB is more inflammogenic than other particulates, probably caused by increased surface area (24–27). The increased production after stimulation with ufCB was not observed in controls. Consequently, these data suggest a different response of PMN to ultrafine particles *in vitro.* PMN of COPD patients react stronger to stimulation with small respirable particles, which suggests that there is a difference between PMN of COPD patients and PMN of healthy persons.

In conclusion, the increased SA release in COPD patients with increasing concentrations of ufCB may suggest that PMN in COPD patients are different from PMN in healthy persons. Furthermore, the smaller particle size of ufCB is a crucial factor in the stimulation of PMN, since similar observations were not made after stimulation with DEP and CB.

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